Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation

Michele A. West*, Alan R. Prescott*, Eeva-Liisa Eskelinen*, Anne J. Ridley† and Colin Watts*

Background: Dendritic cells use constitutive macropinocytosis to capture exogenous antigens for presentation on MHC molecules. Upon exposure to inflammatory stimuli or bacterial products such as lipopolysaccharide (LPS), macropinocytosis is dramatically downregulated as part of a developmental programme leading to dendritic cell maturation, migration and activation of T cells. It is not known, however, how macropinocytosis is sustained in dendritic cells in the absence of exogenous stimuli, nor how it is downregulated upon maturation. We have tested the possibility that one or more members of the Rho family of GTPases are involved in and control pinocytosis in dendritic cells.

Results: We established dendritic cell populations that show constitutive macropinocytosis that was downregulated by LPS treatment. Microinjection of immature cells with dominant-negative Rac (N17Rac1) or treatment with *Clostridium difficile* toxin B, the phosphoinositide 3-kinase (PI3-K) inhibitor wortmannin, or LPS all inhibited the formation of macropinosomes but, surprisingly, did not eliminate membrane ruffling. Microinjection of N17Cdc42 or the Rho inhibitor C3 transferase eliminated actin plaques/podosomes and actin cables, respectively, but had little effect on the formation of macropinosomes. Surprisingly, dendritic cells matured with LPS had equivalent or even somewhat higher levels of active Rac than immature cells. Moreover, microinjection of a constitutively active form of Rac (V12Rac1) into mature dendritic cells did not reactivate macropinocytosis.

Conclusions: Rac has an important role in the constitutive formation of macropinosomes in dendritic cells but may be required downstream of membrane ruffling. Furthermore, regulation of Rac activity does not appear to be the control point in the physiological downregulation of dendritic cell pinocytosis. Instead, one or more downstream effectors may be modulated to allow Rac to continue to regulate other cellular functions.

Addresses: *Department of Biochemistry, MSI/WTB Complex, University of Dundee, Dow Street, Dundee DDI 5EH, UK. †Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, UK.

Correspondence: Colin Watts
E-mail: c.watts@dundee.ac.uk

Received: **13 April 2000**
Revised: **5 June 2000**
Accepted: **6 June 2000**

Published: **26 June 2000**

Current Biology 2000, 10:839–848

0960-9822/00/$ – see front matter

Background

Dendritic cells have a crucial role in the immune response, being unique in their ability to effectively trigger a primary T-cell response to foreign antigen [1]. Dendritic cells exist in distinct developmental states, which allows them to fulfil their roles in antigen capture and presentation. Immature bone-marrow-derived dendritic cells seed the peripheral tissues, sampling and processing the antigens they encounter. In response to local inflammatory stimuli or infectious agents, dendritic cells undergo a maturation process during which they migrate to secondary lymphoid organs where they present previously captured antigenic material to naive T cells. *In vitro* recapitulation of these events has revealed that dendritic cells upregulate the expression of MHC molecules, adhesion molecules and co-stimulatory molecules and downregulate their endocytotic capacity as part of this developmental programme [1–4].

Macropinocytosis has emerged as a key antigen uptake pathway by which dendritic cells can rapidly and non-specifically sample large amounts of surrounding fluid [5–7]. Exogenous antigen taken up by this route can be processed and presented to T cells on both MHC class I and class II molecules [6,7]. Macropinosomes are large (~0.2–5.0 μm diameter) endocytic structures which form when membrane ruffles fuse together, non-selectively engulfing fluid-phase material [8]. Particularly striking in dendritic cells is the fact that membrane ruffling and macropinocytosis are constitutive [6,7], whereas in fibroblasts, epithelial cells and most other cell types studied, these events require stimulation by growth factors [9–12]. Moreover, in cultured human monocyte-derived dendritic cells at least, macropinocytosis is downregulated by inflammatory stimuli [6].

The Rho-family GTPases Rac, Rho and Cdc42 are major regulators of the actin cytoskeleton. In fibroblasts, Rac

activation leads to membrane ruffling and pinocytosis [13]. Parallel roles for Rho in regulating the assembly of focal adhesions and stress fibres and for Cdc42 in the formation of peripheral filopodia have also been identified [14–16]. Subsequently, many other studies have confirmed a role for Rac in stimulating ruffling and lamellipodium extension in a variety of different cell types, including lymphocytes, macrophages, mast cells and neurons. Rac has also been implicated in the regulation of related cellular processes such as adhesion, cell spreading, migration, axonal extension and phagocytosis [15,17–23]. Rac is also known to be a regulatory component of NADPH oxidase in phagocytes and a component of signal transduction pathways linking growth factors to the regulation of gene expression and cell proliferation [24–26].

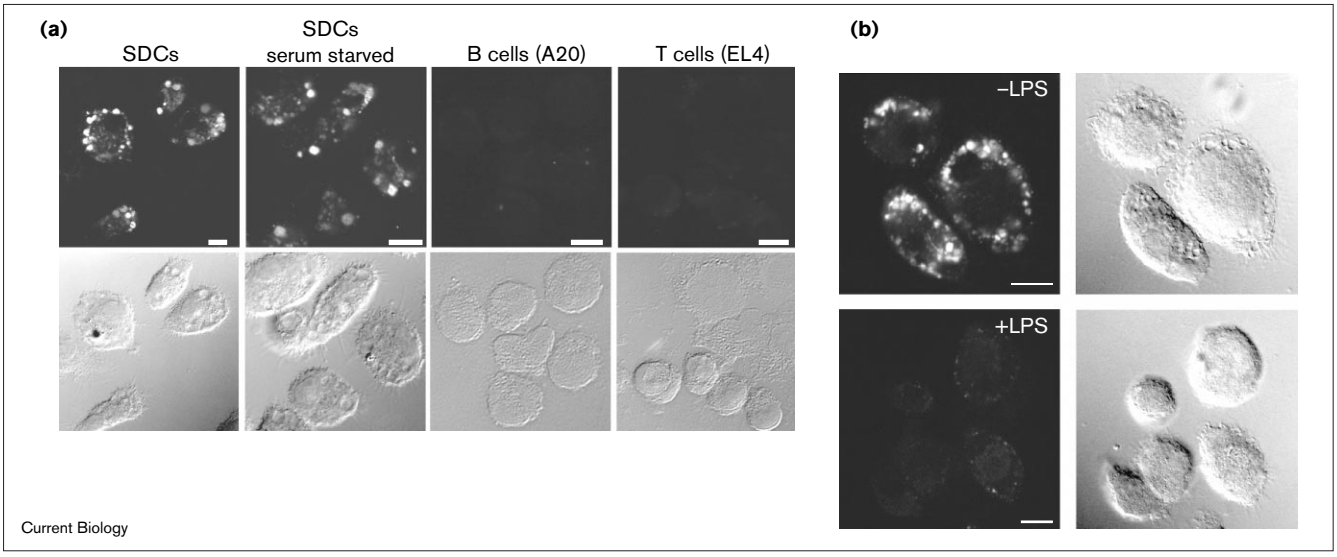
We have investigated two key issues relating to the regulation of macropinocytosis in dendritic cells: first, how immature dendritic cells maintain such high levels of constitutive macropinocytosis, and second, how this activity is downregulated in response to inflammatory stimuli. Specifically, we have investigated the role of Rho-family GTPases in these events. We find a clear requirement for Rac for constitutive macropinocytosis in dendritic cells, but, interestingly, ruffling and formation of macropinosomes appear to be separately regulated. Developmental downregulation of macropinocytosis in this system is not accompanied by a measurable decrease in levels of active Rac, suggesting that one or more downstream elements of the pathway are developmentally regulated.

Results

Spleen-derived murine dendritic cells exhibit high levels of macropinocytosis which can be downregulated by LPS

To investigate the regulation of macropinocytosis in dendritic cells, we have adopted a system for culturing primary spleen-derived dendritic cells (SDCs) in the short to medium term [27]. Murine splenocytes are isolated and cultured in the presence of granulocyte–macrophage colony-stimulating factor (GM–CSF), a cytokine essential for dendritic cell growth, and transforming growth factor-β (TGF-β), which has been shown to support the outgrowth of immature, Langerhans-type dendritic cells [28–30]. After approximately 14 days, the cultures are highly enriched for dendritic cells compared with the starting population, as judged by FACS analysis of expression of markers for B cells, T cells and dendritic cells. The dendritic cell integrin CD11c was almost undetectable in the starting population, but was expressed on the majority of cells cultured in GM–CSF and TGF-β. B and T cells, which were abundant in the starting population, were absent from the final SDC population (see Supplementary material). A large proportion of cells in the starting population (presumably B cells) also expressed high levels of MHC class II molecules, which were much reduced on

Figure 1



Selective downregulation of constitutive macropinocytosis in LPS-treated spleen dendritic cells (SDCs). **(a)** Immature SDCs after 14 days culture (first panels), SDCs that had been starved of growth factors and serum for 3 h (second panels), and the B and T cell lines A20 and EL4 (third and fourth panels, respectively) were incubated with 1.5 mg/ml FITC–dextran for 20 min at 37°C. In the fluorescence micrographs (top panels), macropinosomes are visible as large fluorescently labelled vacuoles in SDCs, but not in B or T cells. Corresponding differential interference contrast (DIC) images are shown in the bottom panels. **(b)** LPS-treated (+LPS) and untreated (–LPS) SDCs (mSDCs and iSDCs, respectively) were analysed for macropinocytosis as in (a). No FITC–dextran-labelled macropinosomes were detectable in mSDCs after 10 min. The scale bars represent 10 μm.

immature SDCs. SDCs also expressed DEC-205 and other markers associated with immature dendritic cells.

To compare the endocytic capacity of SDCs with that of other cell types, we followed the uptake of the fluid-phase marker FITC–dextran. Within minutes, SDCs formed many large (> 300 nm diameter) FITC-labelled macropinosomes (Figure 1a). Remarkably, removal of GM–CSF, TGF-β and serum for 3 hours did not abolish macropinocytosis (Figure 1a), confirming that this process is constitutive in immature murine SDCs, as it is in human monocyte-derived dendritic cells [6]. In contrast, B and T lymphocytes showed no evidence of FITC uptake into macropinocytic structures over this time scale, although smaller labelled structures, probably representing conventional endocytic vesicles, were just detectable (Figure 1a).

Immature dendritic cells can be induced to differentiate into mature dendritic cells by treatment *in vitro* with a variety of inflammatory stimuli or infectious agents, including bacterial products such as LPS [31–34]. To test whether we could drive the maturation of SDCs, we pre-treated the cells for 40 hours with LPS and then assessed their capacity for macropinocytosis. Uptake of FITC–dextran into macropinosomes was dramatically reduced compared with untreated cells demonstrating that our culture system generates dendritic cells that display an authentic LPS-regulated pinocytic response (Figure 1b). We refer to the LPS-treated and untreated cells as mature SDCs (mSDCs) and immature SDCs (iSDCs), respectively. In parallel, we also quantitated the number of clathrin-coated pits and vesicles as markers of the conventional micropinocytic pathway. Whereas iSDCs had 6.0 ± 1.15 coated pits per 100 μm cell surface, on mSDCs this was reduced, but only to a small extent (5.0 ± 0.79 pits per 100 μm cell surface). Thus there is a selective downregulation of macropinocytosis on SDC maturation.

Dominant-negative Rac specifically inhibits macropinocytosis in immature SDCs

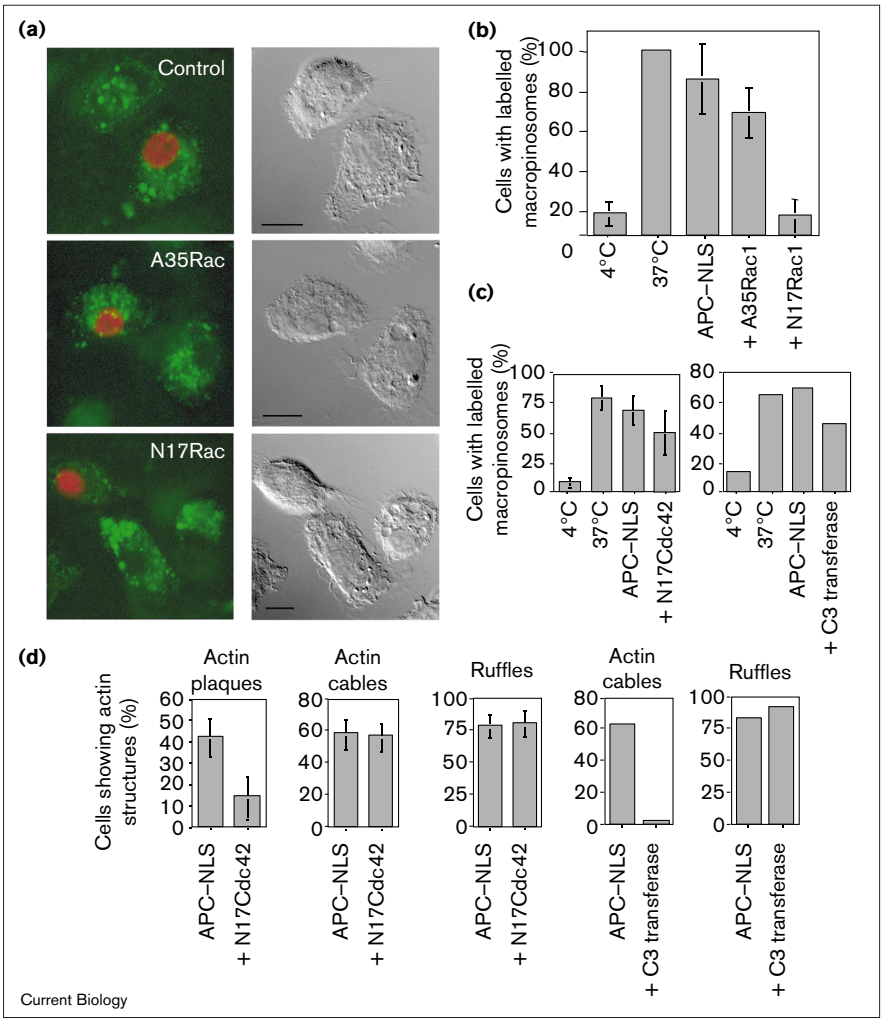
To test the possible role of Rho-family GTPases in the maintenance of constitutive macropinocytosis in SDCs, we first exposed the iSDCs to *Clostridium difficile* toxin B, which has been shown specifically to glucosylate Rac, Rho and Cdc42 and inhibit their function [35]. Macropinocytosis in iSDCs was strongly inhibited under these conditions (only 14% of treated cells had labelled pinosomes compared with 92% of untreated cells), indicating that one or more members of this GTPase family are required to maintain constitutive macropinocytosis. To test the requirement for Rac, Cdc42 and Rho individually we microinjected N17Rac1, N17Cdc42 or *C. botulinum* C3 transferase to block endogenous Rac, Cdc42 and Rho function, respectively [13]. We also established a rigorous new test to ensure that only viable cells were analysed after microinjection. The naturally fluorescent protein allophycocyanin (APC) was conjugated to a nuclear localisation signal peptide (NLS) and co-injected into the cytosol. APC–NLS translocates into and accumulates in the nucleus in an energy-dependent manner, providing not just a marker for identifying injected cells, but also an indicator of cell viability following microinjection. Only cells with clearly fluorescent nuclei were considered in these experiments. After microinjection, the cells were incubated at 37°C for 20 minutes and then exposed to FITC–dextran for 10 minutes to label newly forming macropinosomes.

Cells injected with APC–NLS alone, or with APC–NLS and the inactive mutant A35Rac1 [13] as controls, exhibited large FITC-labelled pinosomes, also seen in neighbouring non-injected cells (Figure 2a, top and middle panels). Cells injected with N17Rac1, however, failed to macropinocytose FITC–dextran (Figure 2a, bottom panel). Microinjected cells were scored positive in this assay if they formed one or more FITC-labelled macropinosomes in 10 minutes. Quantitation from four independent experiments, scoring a total of 450 N17Rac1-injected cells, confirmed that N17Rac1 completely inhibited macropinocytosis in immature SDCs (Figure 2b). The Rac-related GTPases Rho and Cdc42 also regulate actin cytoskeleton rearrangements and Rac, Rho and Cdc42 have all been implicated in phagocytosis, another actin-dependent endocytic pathway [22,23,36]. We tested the specific Rho inhibitor C3 transferase and a dominant-negative mutant of Cdc42, N17Cdc42, for their effects on macropinocytosis in iSDCs. As shown in Figure 2c, both inhibitors caused a small reduction in the percentage of injected cells showing labelled macropinosomes. The effects were moderate compared with the more dramatic inhibition of macropinocytosis observed with N17Rac1 (Figure 2b), and may be indirectly due to changes in cell morphology. Both C3 transferase and N17Cdc42 induced clear-cut effects on other actin-based cytoskeletal structures in SDCs, however. Microinjection with N17Cdc42 reduced or abolished clusters of distinct actin plaques observed in SDCs whereas microinjection of recombinant C3 transferase specifically eliminated actin cables. Neither of these inhibitors affected the presence of membrane ruffles. Quantitation of these effects of C3 transferase and N17Cdc42 is shown in Figure 2d; representative images used for this analysis can be seen in Supplementary material.

Persistence of membrane ruffles in the absence of macropinosome formation

Because active Rac is required for growth-factor-stimulated membrane ruffling in a number of cell types and for constitutive macropinocytosis in SDCs, it seemed likely that N17Rac1 inhibited macropinocytosis in iSDCs by inhibiting the formation of membrane ruffles, which are the precursors of macropinosomes. To test this, we

Figure 2

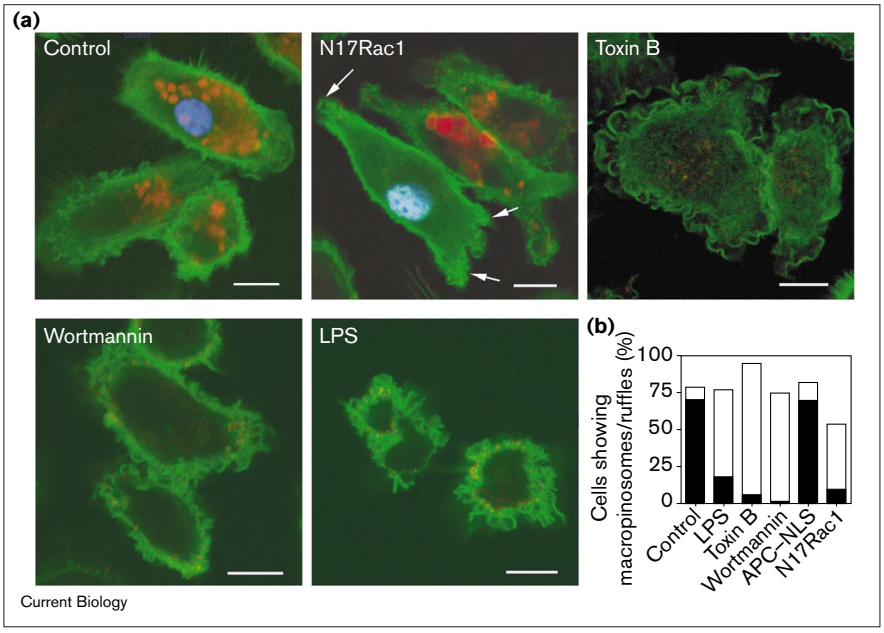


Rac is specifically required for constitutive macropinocytosis in SDCs. **(a)** iSDCs were microinjected with APC–NLS alone (top), or with APC–NLS and A35Rac1 (middle) or N17Rac1 (bottom), then exposed to FITC–dextran for 10 min at 37°C. Cells that had taken up APC–NLS into the nucleus (shown in red) were scored for the presence or absence of any FITC-labelled macropinosomes (green). DIC images of the same cells are shown on the right. **(b)** Quantitation of the results shown in (a) from four independent experiments scoring a total of 450 N17Rac1-injected cells. The percentage of non-injected cells showing labelled macropinosomes after incubation at either 4°C or 37°C is also shown. The effect of microinjected N17Cdc42 and C3 transferase on **(c)** constitutive macropinocytosis and **(d)** actin plaques, cables and ruffles, was assessed following microinjection. Data are expressed as the mean of three independent experiments for N17Cdc42 (540 injected cells scored) or shows a representative experiment of two performed for C3 transferase (180 cells scored). Representative images of cells used to collect the data in (d) can be viewed in Supplementary material. The scale bar in (a) represents 10 μm.

performed double-labelling experiments in which phalloidin-labelled actin structures and macropinosomes containing FITC–dextran could be observed in the same injected cells. Surprisingly, although N17Rac1-injected iSDCs were essentially devoid of macropinosomes, they still displayed actin-rich membrane ruffles (Figure 3a). Control experiments clearly showed that the same preparation of N17Rac1, when injected into quiescent Swiss 3T3 fibroblasts, effectively blocked ruffling in response to phorbol ester stimulation (data not shown). As an alternative means of inhibiting Rac function, we treated iSDCs with toxin B for 30 minutes and then assessed the same cells for the presence of ruffles and labelled macropinosomes. Membrane ruffles were still strikingly evident on cells treated with toxin B, although the morphology of the ruffles was somewhat different (Figure 3a). Nonetheless, macropinosome formation was completely abolished (Figure 3a). Thus, constitutive ruffling in iSDCs appears to be much less dependent on Rac function than in other cell types, in which exogenous stimuli are used to induce it.

PI3-K has been implicated in growth-factor-stimulated ruffling and fluid-phase pinocytosis [37–40]. When we tested the effects of the PI3-K inhibitor wortmannin on ruffling and macropinocytosis in SDCs, we made essentially the same observation: macropinocytosis was virtually eliminated, whereas phalloidin-stained membrane ruffles could still be seen in the same treated cells (Figure 3a). Finally, membrane ruffles or veils were still abundant, or indeed more pronounced, under conditions where macropinocytosis was inhibited by LPS-driven SDC maturation (Figure 3a). The effects of N17Rac1, toxin B, wortmannin and LPS on the presence of membrane ruffles and labelled macropinosomes were quantitated in the same cells. The results clearly show that while all four treatments inhibited macropinocytosis, membrane ruffles persisted, indicating the existence of

Figure 3



Membrane ruffles persist in the absence of macropinosome formation in SDCs. iSDCs were microinjected with APC–NLS alone or with APC–NLS together with N17Rac1, or were treated with toxin B or wortmannin, or induced to mature with LPS. **(a)** SDCs were exposed to FITC–dextran for 10 min to label macropinosomes (shown in red), then fixed and stained for actin with TRITC–phalloidin (shown in green). Microinjected cells have blue/cyan nuclei. Persisting ruffles in injected cells are indicated with arrows. **(b)** For each condition, the percentage of cells that displayed actin-rich ruffles was determined (white bars), and among those cells, the percentage also containing any labelled macropinosomes was assessed (black portions of the bars). The scale bars in (a) represent 10 μm.

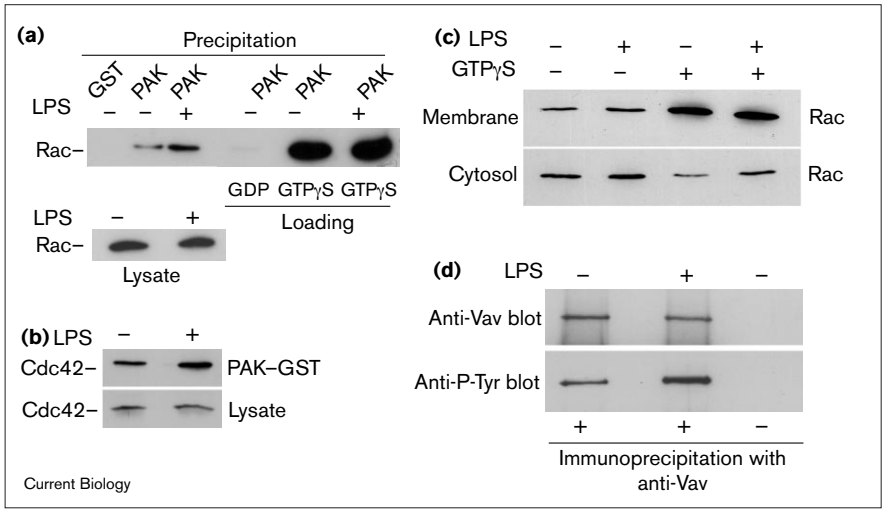
additional points of regulation for macropinocytosis in SDCs (Figure 3b).

Levels of active Rac, Cdc42 and Vav in iSDCs versus mSDCs

Because active Rac is required for constitutive macropinocytosis in iSDCs, a potential mechanism for the downregulation of pinocytosis in mature cells would be through a reduction in levels of Rac protein or Rac–GTP. To test this hypothesis, we used a fusion protein comprising a p21-activated kinase (PAK) CRIB domain fused to glutathione-*S*-transferase (GST), which specifically binds Rac–GTP and Cdc42–GTP but not the inactive GDP-bound forms, to directly measure levels of active Rac in lysates from LPS-treated and untreated SDCs [41–43]. As shown in Figure 4a, when Rac present in SDC lysates was loaded with GDP or the poorly hydrolysable GTP analogue GTPγS, the GST–PAK fusion protein selectively precipitated GTP-bound rather than GDP-bound Rac. We then prepared lysates from iSDCs and mSDCs and performed Rac affinity precipitations using the GST–PAK fusion protein. Rac could clearly be precipitated from iSDC lysates with GST–PAK, whereas in a control precipitation with GST alone, no Rac was detected by western blotting (Figure 4a). When we carried out a precipitation from mSDC, however, rather than showing a reduced amount of active Rac, LPS-matured SDCs had two- to threefold higher levels of PAK-precipitable Rac (Figure 4a). Total Rac levels in the lysates from iSDCs and mSDCs were essentially unchanged (Figure 4a, lower panel). The overall consensus from seven experiments, each of which showed efficient LPS-driven downregulation of macropinocytosis, was that Rac activity either increased or remained essentially the same. This striking result indicates that whereas Rac activity is clearly required for constitutive macropinocytosis, this does not appear to be the control point for the developmental regulation of macropinocytosis. A similar analysis was performed for Cdc42, which also interacts with the PAK CRIB domain [44]. As shown in Figure 4b, there was also a detectable and reproducible small increase in the amount of precipitable Cdc42 in LPS-matured cells.

We also assessed two other potential indicators of Rac activity, membrane association of Rac and levels of the guanine-nucleotide exchange factor (GEF) Vav. We fractionated LPS-treated and untreated SDCs into membrane/cytoskeleton and cytosolic pools, and quantitated the levels of Rac in each by western blotting. A small percentage of total Rac was recovered in the membrane/cytoskeleton fraction in iSDCs, but could be increased fivefold by incubating the lysates in the presence of GTPγS (Figure 4c). Levels of membrane-associated Rac in samples from LPS-treated SDCs were virtually unchanged, however, (11% of total after subtraction of background due to cytosol contamination) compared with untreated samples (12%), supporting the previous observation that Rac–GTP levels were not reduced in LPS-matured cells (Figure 4a). Vav is expressed in haematopoietic cells [45] and is known to stimulate nucleotide exchange on Rac [46]. Vav activity is dependent on tyrosine phosphorylation by the Src-family kinase Lck, and can be regulated both directly and indirectly by products of PI3-K [47]. We compared levels of total and tyrosine-phoshorylated Vav immunoprecipitated

Figure 4



Levels of active Rac and Cdc42 are maintained on LPS-driven maturation of SDC. **(a)** To determine Rac, iSDCs (– LPS) or mSDCs (+ LPS) were lysed (10^7 cells/sample) and affinity precipitated with GST–PAK (PAK) or control GST (upper blot). Bound proteins were probed on western blots with an anti-Rac antibody. Control lysates were loaded with GDP or GTPγS before GST–PAK precipitation. The lower blot shows the analysis of 3% of a lysate for levels of total Rac protein. **(b)** Determination of Cdc42. Experiments were carried out as for (a), except that 1.7×10^7 cells were lysed for each sample and western blots were probed with an anti-Cdc42 antibody. **(c)** Lysates of iSDCs (– LPS) or mSDCs (+ LPS) were incubated with or without GTPγS, and then fractionated into fractions enriched in membrane and cytosol, respectively. Levels of Rac were assessed by western blotting following electrophoresis of 100% of the membrane fraction and 30% of the cytosol fraction. Quantitation by densitometry indicated that 17% and 20% of total recovered Rac was associated with membrane fractions of iSDC and mSDC, respectively. Parallel analysis of a cytosolic marker (AMP-activated kinase) indicated 5% and 9% contamination of the respective membrane fractions by cytosol (data not shown). **(d)** Vav protein in lysates of iSDC (–LPS) or mSDC (+LPS) was immunoprecipitated with anti-Vav or control antibody (iSDC only, rightmost lanes) and the western blots probed with specific antibodies for total Vav protein or for tyrosine-phosphorylated Vav (P-Tyr).

from lysates of immature or LPS-treated SDCs, and found that although total Vav was slightly reduced, the extent of phosphorylation, and therefore the level of potentially active Vav, was actually elevated in mature cells (Figure 4d). Thus, neither the activity of Rac, nor its membrane localisation, nor the level of the active form of at least one GEF for Rac are downregulated upon dendritic cell maturation, in spite of the fact that Rac-dependent macropinocytosis is completely shut down.

Constitutively active Rac does not overcome the downregulation of macropinocytosis in mature SDCs

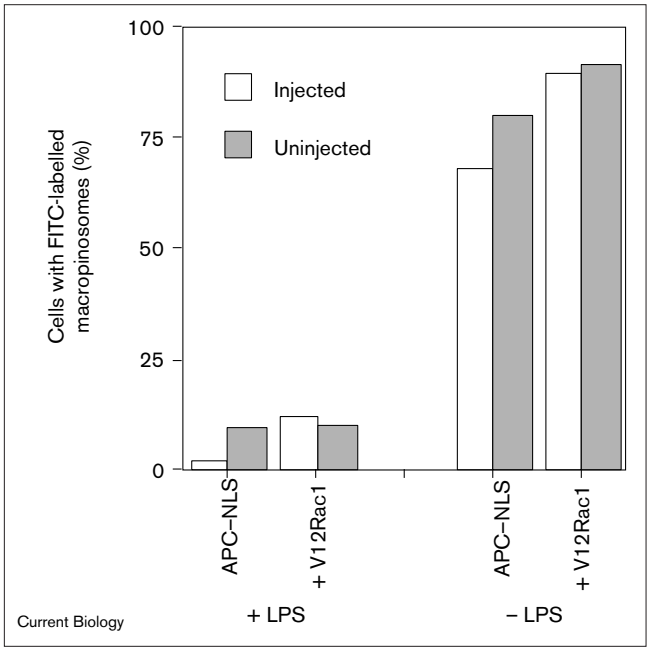
To strengthen our conclusion that downregulation of pinocytosis in mature SDCs is not controlled by downregulation of Rac activity, we microinjected a constitutively active mutant of Rac (V12Rac1) into LPS-treated SDCs. V12Rac1 stimulates ruffling and macropinocytosis in fibroblasts [13]. After a short incubation period, we exposed the injected cells to FITC–dextran to assay macropinosome formation. As shown in Figure 5, there was no significant increase in macropinosome formation in V12Rac1-injected mature cells compared with neighbouring uninjected cells, and levels of macropinocytosis remained far below those in untreated iSDCs. The preparation of V12Rac1 was fully active, as judged by its ability to induce membrane ruffles in 85% of quiescent Swiss 3T3 fibroblasts. V12Cdc42 was also unable to restimulate macropinocytosis in mature SDCs (data not shown). Thus downregulation of constitutive macropinocytosis does not appear to be induced by decreased Rac activity in immature SDCs, nor could it be reactivated in mature SDCs by manipulation of the levels of active Rac.

Discussion

Although the significance of macropinocytosis in many cell types is not obvious, it has considerable physiological relevance in dendritic cells, enabling the non-specific sampling of proteins in the external milieu and display of processed peptides on MHC molecules. Furthermore, this feature of the immune-stimulatory arsenal of dendritic cells is subject to unusual regulation, being constitutive in immature cells and downregulated upon cell maturation. This makes biological sense, distinguishing a phase of antigen capture in peripheral tissues from a phase of antigen presentation after dendritic cell migration to lymphoid tissue [1]. The persistence of MHC–peptide complexes formed in immature cells is likely to be aided by low rates of endocytosis and membrane turnover [6].

To study this regulation we have used a method of culturing murine SDCs using GM–CSF and TGF-β [27]. This system has several key features for our purpose. First, populations of immature dendritic cells are obtained, of which ~90% show very active macropinocytosis. Second, using LPS, we can simulate physiological maturation and effectively downregulate macropinocytosis in virtually all cells. Third, the use of *ex vivo* murine cells allows the use of gene-targeted mice to probe dendritic cell function. For

Figure 5



The constitutively active Rac mutant V12Rac1 does not rescue macropinocytosis in LPS-matured dendritic cells. iSDCs (– LPS) or mSDCs (+ LPS) were microinjected with APC–NLS with or without V12Rac1 protein. The cells were exposed to FITC–dextran for 10 min and viable injected cells and neighbouring non-injected cells were scored for the presence of any labelled macropinosomes (77 V12Rac1-injected cells were scored). Note the failure of V12Rac1 to induce macropinocytosis in LPS-treated cells over the background levels seen in surrounding non-injected cells.

example, we recently showed that the actin-severing and capping protein gelsolin is not required for active ruffling and macropinocytosis in cultured SDCs [27].

Membrane ruffling and macropinocytosis are constitutive in SDCs, continuing for several hours after GM–CSF, TGF-β and serum have been removed. This raised the question of whether Rho-family GTPases would regulate these activities, as they do exogenously stimulated membrane ruffling and macropinocytosis in other cells. Systematic testing of dominant-negative mutants or inhibitors of Rac, Cdc42 and Rho established that there is a requirement for Rac function in constitutive macropinocytosis in dendritic cells. Dominant-negative mutants of Rho-family GTPases are believed to function by sequestering GEFs, and therefore preventing the activation of endogenous GTPases [48]. As some GEFs can activate more than one Rho family member, at least *in vitro*, the possibility that an inhibitory GTPase may actually block activation of other related GTPases should be considered. In our system, however, we find that inhibitors of Rac, Rho and Cdc42 produce distinct effects on the actin cytoskeleton and macropinocytosis in dendritic cells, suggesting that N17Rac1 does not inhibit macropinocytosis by inhibiting activation of Rho or Cdc42. N17Cdc42 was a much less effective inhibitor of macropinosome formation than N17Rac1, in spite of being able to substantially reduce actin plaques; similarly, C3 transferase, an independent inhibitor of Rho, only slightly reduced macropinocytosis while specifically and completely abolishing actin cables.

Because membrane ruffling is clearly a prerequisite for macropinocytosis, and Rac is strongly implicated in regulating ruffling, an unexpected finding was that although N17Rac1 abolished macropinocytosis in SDCs it did not eliminate membrane ruffles. Indeed, ruffles also persisted on iSDCs treated with toxin B and wortmannin and on LPS-matured mSDCs, all of which had downregulated macropinocytotic activity (Figure 3). There are various possible explanations. First, there might be different types of ruffles that are differentially dependent on Rac function and differ in their ability to contribute to macropinosome formation. Membrane ruffles and lamellipodia, generally defined as cell-surface protrusions containing a meshwork of newly polymerised actin filaments, can take on a variety of appearances depending on cell type and conditions [49], and there is evidence that not all ruffles are structurally identical; for example, some ruffles on chick embryo fibroblasts are distinguishable from others by their dependence on microtubules [50]. An alternative possibility might be that in SDCs, Rac is required for the conversion or closure of membrane ruffles to form macropinosomes, rather than being required for ruffling *per se*. In fact, the idea of regulation of macropinocytosis at a point downstream of ruffling has already been proposed for macrophages, where, as we also find for SDCs, the PI3-K inhibitor wortmannin appeared to block a late step in macropinosome formation, and not ruffling as such [51]. Finally, our data do not exclude the possibility that downstream targets of Rac involved in constitutive ruffling in other cell types are constitutively activated in SDCs, or could be activated by alternative mechanisms, perhaps involving members of the Rho family other than Rac, Cdc42 and Rho.

Although ruffling appeared to be independent of Rac in SDCs, our data nonetheless indicate that Rac is a key regulator of macropinocytosis in immature SDCs, leading us to speculate that macropinosome formation might be downregulated in mature cells through a reduction in Rac levels or activity. We found, however, that Rac–GTP levels were unchanged, or actually increased, in mature dendritic cells compared to immature cells. Furthermore, the introduction of constitutively active Rac could not restore macropinocytosis in mature SDCs. These data suggest that macropinocytosis is downregulated, at least in cultured SDCs, by some other mechanism. One possibility is that one or more effectors of Rac, or elements of the actin cytoskeleton required for pinosome formation, are

differentially expressed in immature versus mature dendritic cells. Alternatively, the signalling pathway may be modified in mature cells at the level of upstream regulators of Rac, for example by expression of different GEFs, which could affect the specificity of downstream events without greatly changing net levels of active Rac [52].

Garrett and colleagues have recently completed a similar analysis of developmental regulation of endocytosis in murine dendritic cells derived from bone marrow rather than spleen (I. Mellman, personal communication). In this system, regulation of endocytosis appears to be controlled by the levels of active Cdc42. Indeed, macropinocytosis in mature cells could be rescued by microinjection of active Cdc42. The reasons for the differences in these two studies are not yet clear; it is, however, important to note that both the source of dendritic cell precursors and the culture conditions were different. Our spleen cultures were usually 14–28-day cells cultured with GM–CSF and TGF-β whereas the bone-marrow cultures were shorter term and did not include TGF-β. TGF-β is known to be required for outgrowth of Langerhans-type dendritic cells, for example, from $CD34^+$ precursors [29,30,53], although we do not know how closely our cells resemble Langerhans cells. Conceivably, the spleen and bone-marrow dendritic cell populations may differentially express Rho GTPases and/or effectors, which could account for the differences in regulation. Taken together, these data indicate that dendritic cells might use different strategies to regulate endocytosis in response to environmental stimuli. As Rho-family GTPases are, however, likely to be required for events such as dendritic cell migration and interaction with T cells, which take place downstream of the early phase of constitutive endocytosis, it may be advantageous to maintain active GTPases and instead modulate downstream effector systems. Analysis of the changes in gene expression that take place on dendritic cell maturation may provide further information.

Materials and methods

Cell culture

Dendritic cell populations from the spleens of 8–16-week-old female C57BL/6 mice were expanded in low-attachment six-well plates (Corning-Costar) in the presence of 20 ng/ml GM–CSF and 1 ng/ml TGF-β as described previously [27]. Cells were generally used after 14–28 days of culture. Where indicated, the cultures were treated with LPS (*Escherichia coli* 026:B6; Sigma) at 1 μg/ml for 40 h to induce maturation. A representative FACS analysis of day 0 spleen and the day 14 immature DC population can be seen in Supplementary material. The mouse B-lymphoma line A20 and thymoma line EL4 were maintained in RPMI medium supplemented with 10% FCS, 1 mM pyruvate, 1× non-essential amino acids, 2 mM glutamine, 100 μg/ml kanamycin and 50 μM β-mercaptoethanol (complete RPMI).

Fusion proteins and probes

Recombinant GST fusion proteins with V12Rac1, V12A35Rac1, V12N17Rac1, N17Cdc42, *C. botulinum* C3 transferase (a gift from L. Feig) and the PAK CRIB domain (human PAK1B amino acids 59–272, kindly provided by A. Hall) were expressed and purified essentially as described in [13], with the following exceptions. For GST–PAK purification, the bacterial lysates additionally contained 0.5% Triton X-100 and 10% glycerol, and GST–PAK was eluted from the glutathione–sepharose beads with 15 mM glutathione, before dialysis against 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 10% glycerol. The GST moiety of the other fusion proteins was removed by digestion while still bound to the glutathione–agarose beads, in 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM DTT, with bovine thrombin (Sigma) at 5 U per ml of beads for 1 h at room temperature. The supernatants that contained the GST-free proteins were treated with *p*-aminobenzamidine–agarose (Sigma) to remove thrombin, before dialysis against 100 mM KCl, 20 mM Hepes pH 7.3, 2.5 mM $MgCl_2$, 1 mM DTT (microinjection buffer) and concentration. All fusion proteins were snap-frozen in small aliquots and stored at –80°C.

Allophycocyanin (Calbiochem) was chemically coupled to a synthetic peptide containing the SV40 large T antigen nuclear localisation signal (CGGGPKKKRKVED) as described [54], dialysed into microinjection buffer and stored as small aliquots at –80°C.

Microinjection

SDCs (2.5×10^5) were plated on 13 mm glass coverslips in complete RPMI medium for 2–3 h before microinjection. The cells were co-injected in the cytoplasm with APC–NLS at 2 mg/ml and recombinant proteins, as indicated, at the following total protein concentrations: V12Rac1 (1.3 mg/ml), V12A35Rac1 (0.4 mg/ml), V12N17Rac (0.4 mg/ml), N17Cdc42 (0.7 mg/ml), C3 transferase (2 μg/ml). Approximately 150 cells per coverslip were injected in 10 min using an Eppendorf Transjector 5246 and a Zeiss Axiovert 100 microscope fitted with a 37°C heated chamber. The cells were further incubated for 20 min at 37°C before FITC–dextran internalisation or fixation and staining for actin.

FITC–dextran uptake and fluorescence microscopy

Macropinocytosis was assessed by FITC–dextran uptake as described previously [27]. For the non-adherant A20 and EL4 cell lines, 2×10^5 cells were centrifuged onto slides at 200 rpm for 2 min (Shandon Cytospin 3) before fixation. Where indicated, cells were treated for 30 min with 100 ng/ml toxin B before uptake, or were pretreated for 15 min with 100 nM wortmannin (Alexis Biochemicals), then wortmannin was included in the FITC–dextran incubation. Actin structures were visualised after fixation and staining with TRITC–phalloidin as described previously [27]. Images were collected on a Zeiss LSM 410 laser scanning microscope using a ×63, NA1.4 Planapochromatic objective.

Affinity precipitation, immunoprecipitation and cell fractionation

For affinity precipitation of active Rac and Cdc42, SDCs were resuspended at 10^7cells/ml in 100 mM NaCl, 25 mM Tris-HCl pH 7.5, 1% NP-40, 5% glycerol, 5 mM $MgCl_2$ containing leupeptin (50 μM), pepstatin A (10 μM), chymostatin (10 μg/ml) and AEBSF (100 μg/ml; ICN). GST or GST–PAK (25 μg) was added immediately to each sample. After lysis for 15 min at 4°C and centrifugation at 2500 *g* for 5 min to remove debris, 25 μl of glutathione–sepharose suspension was added, and the samples incubated for 30 min at 4°C with mixing. Control lysates were treated with 10 mM EDTA for 20 min at 30°C in the presence of 1 mM GDP or 0.5 mM GTPγS, then $MgCl_2$ was added to 30 mM before precipitation with GST–PAK and glutathione–sepharose. Precipitated complexes were washed four times with lysis buffer, then analysed by western blotting. Rac and Cdc42 were detected using monoclonal antibody 23A8 and polyclonal anti-Cdc42 (Santa Cruz), respectively. For immunoprecipitation of Vav, SDCs (8×10^6) were lysed in 40 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% Triton X-100, containing phosphatase inhibitors NaF (50 mM), $Na_4P_2O_7$ (30 mM), Na_3VO_4 (1 mM) and protease inhibitors as above. Lysates were immunoprecipitated with 5 μg anti-Vav polyclonal antibody (UBI) and protein A–sepharose and total Vav or tyrosine-phosphorylated Vav was detected by western blotting using the same anti-Vav antibody or monoclonal PY-20 (Transduction Labs). Detection with PY-20 could be completely competed by the inclusion of 2.5 mM *o*-phosphotyrosine (data not shown).

For membrane fractionation, SDCs (5×10^6/sample) were resuspended in lysis/breakage buffer [55], broken by 10 passages through a ball-bearing homogeniser, and post-nuclear supernatants (PNS) prepared by centrifugation at $1000 \times g$ for 10 min. In control samples, PNS were incubated with 1 mM GTPγS for 10 min at 37°C. Membrane and cytosol fractions were separated at $100{,}000 \times g$ for 20 min at 4°C, and the membranes washed once in lysis/breakage buffer to remove contaminating cytosolic proteins. Rac levels in each fraction were measured by western blotting.

Supplementary material

Supplementary material including a FACS analysis of a representative SDC population is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We are grateful to Chris Norbury both for pilot studies and for advice on the SDC culture method. We thank A. Lamond for access to confocal microscopy facilities, A. Hall for the GST–PAK construct, L. Feig for GST–C3 transferase and D. Kwiatkowski for the 23A8 antibody. We thank Wendy Garrett and Ira Mellman for discussions and communication of unpublished results and A. Antoniou for comments on the manuscript. This work was supported by a Project Grant from the Medical Research Council, an MRC Cooperative Group Grant and anEU Biomed grant.

References

1. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392:**245-252.
2. Cella M, Sallusto F, Lanzavecchia A: **Origin, maturation and antigen presenting function of dendritic cells**. *Curr Opin Immunol* 1997, **9:**10-16.
3 Watts C, Amigorena S: **Antigen traffic pathways in dendritic cells**. *Traffic* 2000, **1:**312-317
4. Mellman I, Turley S, Steinman R: **Antigen presentation for amateurs and professionals.** *Trends Cell Biol* **8:**231-237
5. Steinman RM, Swanson J: **The endocytic activity of dendritic cells.** *J Exp Med* 1995, **182:**283-288.
6. Sallusto F, Cella M, Danieli C, Lanzavecchia A: **Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products**. *J Exp Med* 1995, **182:**389-400.
7. Norbury CC, Chambers BJ, Prescott AR, Ljunggren HG, Watts C: **Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells**. *Eur J Immunol* 1997, **27:**280-288.
8. Swanson JA, Watts C: **Macropinocytosis**. *Trends Cell Biol* 1995, **5:**424-428.
9. Haigler HT, McKanna JA, Cohen S: **Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor** *J Cell Biol* 1979, **83:**82-90.
10. West MA, Bretscher MS, Watts C: **Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells** *J Cell Biol* 1989, **109:**2731-2739.
11. Racoosin EL, Swanson JA: **Macrophage colony-stimulating factor (rM-CSF) stimulates pinocytosis in bone marrow-derived macrophages**. *J Exp Med* 1989, **170:**1635-1648.
12. Veithen A, Cupers P, Baudhuin P, Courtoy PJ: **v-Src induces constitutive macropinocytosis in rat fibroblasts**. *J Cell Sci* 1996, **109:**2005-2012.
13. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A: **The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling**. *Cell* 1992, **70:**401-410.
14. Ridley AJ, Hall A: **The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors**. *Cell* 1992, **70:**389-399.
15. Nobes CD, Hall A: **Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia**. *Cell* 1995, **81:**53-62.
16. Kozma R, Ahmed S, Best A, Lim L: **The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts**. *Mol Cell Biol* 1995, **15:**1942-1952.
17. Arrieumerlou C, Donnadieu E, Brennan P, Keryer G, Bismuth G, Cantrell D, *et al.*: **Involvement of phosphoinositide 3-kinase and Rac in membrane ruffling induced by IL-2 in T cells**. *Eur J Immunol* 1998, **28:**1877-1885.
18. Allen WE, Jones GE, Pollard JW, Ridley AJ: **Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages**. *J Cell Sci* 1997, **110:**707-720.
19. Guillemot JC, Montcourrier P, Vivier E, Davoust J, Chavrier P: **Selective control of membrane ruffling and actin plaque assembly by the Rho GTPases Rac1 and CDC42 in FcεRI-activated rat basophilic leukemia (RBL-2H3) cells**. *J Cell Sci* 1997, **110:**2215-2225.
20. van Leeuwen FN, Kain HET, van der Kammen RA, Michiels F, Kranenburg OW, Collard JG: **The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho**. *J Cell Biol* 1997, **139:**797-807.
21. Kozma R, Sarner S, Ahmed S, Lim L: **Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid**. *Mol Cell Biol* 1997, **17:**1201-1211.
22. Caron E, Hall A: **Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases**. *Science* 1998, **282:**1717-1721.
23. Massol P, Montcourrier P, Guillemot JC, Chavrier P: **Fc receptor-mediated phagocytosis requires CDC42 and Rac1**. *EMBO J* 1998, **17:**6219-6229.
24. Segal AW, Abo A: **The biochemical basis of the NADPH oxidase of phagocytes**. *Trends Biochem Sci* 1993, **18:**43-47.
25. Aspenstrom P: **Effectors for the Rho GTPases**. *Curr Opin Cell Biol* 1999, **11:**95-102.
26. Mackay DJ, Hall A: **Rho GTPases**. *J Biol Chem* 1998, **273:**20685-20688.
27. West M, Antoniou A, Prescott A, Toshifumi A, Kwiatkowski D, Watts C: **Membrane ruffling, macropinocytosis and antigen presentation in the absence of gelsolin in murine dendritic cells**. *Eur J Immunol* 1999, **29:**3450-3455.
28. Yamaguchi Y, Tsumura H, Miwa M, Inaba K: **Contrasting effects of TGF-β1 and TNF-α on the development of dendritic cells from progenitors in mouse bone marrow**. *Stem Cells* 1997, **15:**144-153.
29. Geissmann F, Revy P, Regnault A, Lepelletier Y, Dy M, Brousse N, *et al.*: **TGF-β1 prevents the noncognate maturation of human dendritic Langerhans cells**. *J Immunol* 1999, **162:**4567-4675.
30. Strobl H, Knapp W: **TGFβ1 regulation of dendritic cells**. *Microbes Infect* 1999, **1:**1283-1290.
31. Sallusto F, Lanzavecchia A: **Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus IL4 and downregulated by TNFα**. *J Exp Med* 1994, **179:**1109-1118.
32. Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A: **Maturation, activation, and protection of dendritic cells induced by double-stranded RNA**. *J Exp Med* 1999, **189:**821-829.
33. Romani N, Reider D, Heuer M, Ebner S, Kampgen E, Eibl B, *et al.*: **Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability**. *J Immunol Methods* 1996, **196:**137-151.
34. Reis e Sousa C, Sher A, Kaye P: **Role of dendritic cells in the induction and regulation of immunity to microbial infection.** *Curr Opin Immunol* 1999, **11:**392-399
35. Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K: **Glucosylation of Rho proteins by *Clostridium difficile* toxin B**. *Nature* 1995, **375:**500-503.
36. Hackam DJ, Rotstein OD, Schreiber A, Zhang W-J, Grinstein S: **Rho is required for the initiation of calcium signaling and phagocytosis by Fcγ receptors in macrophages**. *J Exp Med* 1997, **186:**955-966.
37. Wennstrom S, Hawkins P, Cooke F, Hara K, Yonezawa K, Kasuga M, *et al.*: **Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling**. *Curr Biol* 1994, **4:**385-393.
38. Barker SA, Caldwell KK, Hall A, Martinez AM, Pfeiffer JR, Oliver JM, *et al.*: **Wortmannin blocks lipid and protein kinase activities associated with PI 3-kinase and inhibits a subset of responses induced by FcεR1 cross-linking**. *Mol Biol Cell* 1995, **6:**1145-1158.
39. Clague MJ, Thorpe C, Jones AT: **Phosphatidylinositol 3-kinase regulation of fluid phase endocytosis**. *FEBS Lett* 1995, **367:**272-274.
40. Hawkins PT, Eguinoa A, Qiu RG, Stokoe D, Cooke FT, Walters R, *et al.*: **PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase**. *Curr Biol* 1995, **5:**393-403.

41. Glaven JA, Whitehead I, Bagrodia S, Kay R, Cerione RA: **The Dbl-related protein, Lfc, localizes to microtubules and mediates the activation of Rac signaling pathways in cells**. *J Biol Chem* 1999, **274:**2279-2285.
42. Bagrodia S, Taylor SJ, Jordan KA, Van Aelst L, Cerione RA: **A novel regulator of p21-activated kinases**. *J Biol Chem* 1998, **273:**23633-23636.
43. Sander EE, van Delft S, ten Klooster JP, Reid T, van der Kammen RA, Michiels F, *et al.*: **Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase**. *J Cell Biol* 1998, **143:**1385-1398.
44. Thompson G, Owen D, Chalk PA, Lowe PN: **Delineation of the Cdc42/Rac-binding domain of p21-activated kinase**. *Biochemistry* 1998, **37:**7885-7891.
45. Collins TL, Deckert M, Altman A: **Views on Vav**. *Immunol Today* 1997, **18:**221-225.
46. Crespo P, Schuebel KE, Ostrom AA, Gutkind JS, Bustelo XR: **Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product**. *Nature* 1997, **385:**169-172.
47. Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, *et al.*: **Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav**. *Science* 1998, **279:**558-560.
48. Feig LA: **Tools of the trade: use of dominant inhibitory mutants of Ras family GTPases**. *Nat Cell Biol* 1999, **1:**E25-E27.
49. Ridley AJ: **Membrane ruffling and signal transduction**. *BioEssays* 1994, **16:**321-327.
50. Hasegawa T: **Production of a unique antibody specific for membrane ruffles and its use to characterise behaviour of two distinct types of ruffles**. *J Cell Biol* 1993, **120:**1439-1448.
51. Araki N, Johnson MT, Swanson JA: **A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages**. *J Cell Biol* 1996, **135:**1249-1260.
52. Zhou K, Wang Y, Gorski JL, Nomura N, Collard J, Bokoch GM: **Guanine nucleotide exchange factors regulate specificity of downstream signalling from Rac and Cdc42**. *J Biol Chem* 1998, **273:**16782-16786.
53. Jaksits S, Kriehuber E, Charbonnier AS, Rappersberger K, Stingl G, Maurer D: **CD34+ cell derived CD14+ precursor cells develop into Langerhans cells in a TGFβ1-dependent manner**. *J Immunol* 1999, **163:**4869-4877.
54. Adam SA, Sterne-Marr R, Gerace L: **Nuclear protein import using digitonin-permeabilised cells**. *Methods Enzymol* 1992, **219:**97-110.
55. Fleming IN, Elliott CM, Exton JH: **Differential translocation of Rho family GTPases by lysophosphatidic acid, endothelin-1, and platelet-derived growth factor**. *J Biol Chem* 1996, **271:**33067-33073.

Because *Current Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published on the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cub – for further information, see the explanation on the contents page.